Phenotypic antimicrobial susceptibility testing of *Chlamydia trachomatis* isolates from patients with persistent and successfully treated infections

Rachel PITT[1,2]*, Sarah ALEXANDER[2], Catherine ISON[2], Patrick HORNER[3], Emma HATHORN[4], Penny GOOLD[4], Neil WOODFORD[1] and Michelle COLE[1,2]

[1] Antimicrobial Resistance and Healthcare Associated Infections (AMRHAI) Reference Unit, National Infection Service, Public Health England, London, U.K., [2]formerly of The Sexually Transmitted Bacteria Reference Unit, Public Health England, London, U.K., [3]Population Health Sciences, Bristol Medical School, University of Bristol, UK. [4]Whittal Street Clinic, University Hospitals Birmingham NHS Foundation Trust, Birmingham, U.K.

*Corresponding Author Address: Antimicrobial Resistance in Sexually Transmitted Infections (AMRSTI), Antimicrobial Resistance and Healthcare Associated Infections (AMRHAI) Reference Unit, National Infection Service, Public Health England, Colindale, NW9 5EQ

Rachel.pitt@phe.gov.uk

Tel: 020 8327 7339

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Synopsis

Objectives: Antimicrobial susceptibility data for *Chlamydia trachomatis* are lacking. Methodologies for susceptibility testing in *C. trachomatis* are not well-defined, standardised or performed routinely owing to its intracellular growth requirements. We sought to develop an assay for the *in vitro* susceptibility testing of *C. trachomatis* isolates from two patient cohorts with different clinical outcomes.

Methods: Twenty-four clinical isolates (11 from persistently infected and 13 from successfully treated patients) were overlaid with media containing two-fold serial dilutions of azithromycin or doxycycline. After incubation, aliquots were removed from the stock inoculum (SI) and each antimicrobial concentration for total RNA extraction, complementary DNA generation and real-time PCR. The MIC was defined as the lowest antimicrobial concentration where a 95% reduction in transcription was evident in comparison with the SI for each isolate.

Results: MICs of azithromycin were comparable for isolates from the two patient groups (82% ≤0.25 mg/L persistently infected and 100% ≤0.25 mg/L successfully treated patients). Doxycycline MICs were at least two-fold lower for isolates from the successfully treated patients (53.9% ≤0.064 mg/L) than for the persistently infected patients (100% ≥0.125 mg/L) (p=0.006, Fisher's exact test). Overall, 96% of isolates gave reproducible MICs when re-tested.

Conclusions: A reproducible assay was developed for antimicrobial susceptibility testing of *C. trachomatis*. MICs of azithromycin were generally comparable for the two different patient groups. MICs of doxycycline were significantly higher in the persistently infected

patients. However, interpretation of elevated MICs in *C. trachomatis* is extremely challenging in the absence of breakpoints, or wild-type and treatment failure MIC distribution data.

Introduction

Chlamydia trachomatis is the most prevalent bacterial sexually transmitted infection worldwide with 202,546 diagnoses in England in 2016. [1] Current first-line recommended treatment regimens for uncomplicated infection are 1 g stat azithromycin, or 100 mg doxycycline twice a day for seven days. [2] Whilst the efficacy of these treatments is considered to be extremely high, [3, 4] treatment failure with 1 g stat azithromycin has been demonstrated in *C. trachomatis*-positive men with non-gonococcal urethritis and rectal chlamydia and in women not at risk of re-infection. [5-7] Further reports of treatment failure have been described in patients where the risk of re-infection is low. [8-15] There are a number of possible reasons why patients may remain positive for chlamydia after treatment: non-adherence to the treatment regimen; re-infection from a new or untreated partner; inadequate exposure to the antimicrobial as a result of host pharmacokinetics or short duration of treatment, [16] and heterotypic or homotypic antimicrobial resistance. Heterotypic resistance, also known as phenotypic switching, occurs when a heterogeneous population of both resistant and susceptible organisms replicate from a single predecessor. [7] It is not genetically inherited but is a result of adaptations by the bacteria to make them less susceptible to the antimicrobial e.g. induction of slow growing, non-replicative or persistent forms in the presence of antibiotic, which revert back to replicating forms once the antibiotic pressure has been removed resulting in a relapse in infection. Homotypic antimicrobial resistance is, by contrast, genetically inherited.

At high bacterial loads, e.g. as found in patients with symptoms of urethritis, [17] *C. trachomatis* has been shown to exhibit heterotypic resistance. [18, 19] Confirmed phenotypic decreased susceptibility to antimicrobials of clinical significance has been reported rarely in

C. trachomatis.[9, 11, 20, 21] Stable genotypic resistance to antimicrobials in clinical practice has yet to be documented in human urogenital *C. trachomatis* infection.[16] However very little is known about the susceptibility profiles of circulating strains because antimicrobial susceptibility assays are not routinely performed and the methodology is neither standardised nor well-defined.[7, 18, 22]

We describe the development of a robust antimicrobial susceptibility testing methodology (adapted from Storm *et al*[23]), and report susceptibility data for azithromycin and doxycycline for a cohort of *C. trachomatis* isolates from patients who were persistently infected with *C. trachomatis*. Susceptibility data are also presented for *C. trachomatis* isolates from a group of control patients who were *C. trachomatis*-positive at initial presentation and were then confirmed to have been treated successfully.

Methods

Patient recruitment

As reported previously[24] patients with persistent *C. trachomatis* infections were recruited from sexual health clinics across England and Wales. Patients were deemed to have a persistent infection if they had tested positive at least twice by a *C. trachomatis*-specific assay (e.g. a nucleic acid amplification test, NAAT), had fully adhered to the prescribed treatment regimens in line with current guidelines[2] (including any abstinence periods) and were assessed to be at low risk of re-infection. Risk of re-infection was categorised using self-declared sexual contact behaviour in the time since initial diagnosis, reported via a clinician-completed questionnaire. Patients were considered at low risk of re-infection following treatment if they had: a) no sexual contact, b) protected sexual contact only, or c) unprotected sexual contact with a partner who had not tested positive or who had tested positive, but had been treated. These groups were designated categories 1, 2 and 3 respectively. Clinical data collected for some of the patients in this report were reported previously. [24]

Control isolates were collected from patients who had been treated for *C. trachomatis* infection in line with current UK guidelines [2] and had a negative test-of-cure by NAAT at least 30 days later.

Ethical approval

Patients with persistent infections were referred as part of an enhanced surveillance programme and therefore ethical approval was not sought or required. Public Health England has permission to handle these data under the Health Service (Control of Patient Information) regulation 2002, overseen by the Confidentiality Advisory Group.

Control patients were recruited through a sexual health clinic, ethics reference number 13/WM/0088.

Culture methods

Stock inoculum culture

Clinical specimens (persistently infected group: 6 specimens from male patients [5 urethral swabs and 1 rectal swab] and 5 specimens from female patients [4 cervical swabs and 1 urethral swab; successfully treated group: 2 specimens from male patients [1 urethral swab and one swab from an unknown site and 11 specimens from female patients [3 cervical swabs, 8 self-collected vaginal swabs were inoculated on to confluent McCoy cell monolayers in shell vials. Shell vials were centrifuged at 2300 x g for 1 h at 35^oC and were then incubated for 4 h at 35^oC in 5% CO_2. The inoculum was then aspirated and the infected mono-layer was overlaid with Dulbecco's Modified Eagles Medium (DMEM, Gibco, Hemel Hempstead, U.K.) supplemented with 10% foetal bovine serum (Gibco), 200 mM L-glutamine (Sigma, Gillingham, U.K.), 1 mg/L cycloheximide (Sigma), 100 mg/L gentamicin (Gibco), 25 U/mL nystatin (Sigma) and 100 mg/L vancomycin (Sigma). Shell vials were incubated for 48 h at 35^oC in 5% CO_2 to produce a stock inoculum of each strain for antibiotic susceptibility testing assays. Inclusion forming units (IFUs) were visualised after staining with the MicroTrak® *Chlamydia trachomatis* culture confirmation test (Trinity Biotech, Newmarket, U.K.).

Susceptibility assays

MICs of azithromycin and doxycycline were determined as follows; confluent McCoy cell monolayers in 48-well plates were overlaid with the stock inoculum of each strain (10^3-10^5 inclusion forming units per well), plates were centrifuged for 1 h at 1350 x g and 35^oC and were then incubated at 35^oC, 5% CO_2 for 4 h to facilitate infection. Wells were aspirated and overlaid with two-fold serial dilutions of antimicrobial (0.125-2 mg/L azithromycin or 0.064-1 mg/L doxycycline) in supplemented DMEM (as above). An antimicrobial-free control was included for each strain to allow identification of assay failure. Due to the lack of known azithromycin- or doxycycline- resistant control isolates of *C. trachomatis*, a susceptible control *C. trachomatis* isolate (from a successfully treated patient, isolate 314) was used in the azithromycin assays, and the tetracycline-resistant *C. suis* strain R19 [25] was used in the doxycycline assays. Plates were then incubated for 48 h at 35^oC in 5% CO_2.

RNA extractions and cDNA generation

The RNeasy Plus Mini Kit (QIAgen, Manchester, U.K.) was used as per the manufacturer's instructions to extract total RNA from aliquots of culture media collected from each antibiotic concentration and negative control at varying stages during the susceptibility assay, namely the initial inoculum (P0) and after the 48 h incubation with antimicrobial (P1).

Complementary DNA (cDNA) was reverse transcribed from the total RNA (2 µL per reaction) using the Quantitect reverse transcription kit (QIAgen) as per manufacturer's instructions. This kit includes a step for removing contaminating genomic DNA negating the need for a separate *DNase*I digestion.

Real Time-PCR to detect transcription and interpretation of MIC endpoint

cDNA was used as template for real time PCR (RT-PCR) on the RotorGene (QIAgen) platform (primer and probe sequences in Table 1) to quantify the *C. trachomatis* transcripts and allow assignment of an MIC of each antimicrobial for each strain. The method described by Storm *et al* [23] was modified to facilitate use of an L2 internal control, prepared in-house, for transcript quantification. In place of the *omp2* gene target a predicted virulence factor on the *C. trachomatis* cryptic plasmid was used as the chlamydia specific target. [26] The McCoy cell B-actin gene (inhibition control) and the *C. suis* R19 23S rRNA gene were detected qualitatively only where appropriate. To increase assay sensitivity each target was run as a separate reaction.

Twenty-five microliter reactions were prepared for each target in HotStarTaq master mix (QIAgen). Primer and probe sequences can be found in Table 1. *C. trachomatis*-specific target: 200 nM Ct-Forward primer, 320 nM Ct-Reverse primer, 200 nM Ct-Probe and 10 µL cDNA. McCoy cell β-actin-specific target: 100 nM McCoy-Forward primer, 100 nM McCoy-

Reverse primer, 24 nM McCoy-Probe and 5 µL cDNA. *C. suis*-specific target: 200 nM R19-Forward primer, 200 nM R19-Reverse primer, 200 nM R19-Probe and 5 µL cDNA. Reactions were run on the RotorGene platform (QIAgen) using the following programme: initial denaturation and Taq activation step of 95°C for 10 minutes followed by 50 cycles of 95°C for 30 seconds, 60°C for 40 seconds (acquiring in the green [FAM, *C. trachomatis*-specific PCR], yellow [JOE, McCoy cell-specific PCR] or red [Cy5, *C. suis*-specific PCR] channel) and 72°C for 40 seconds. A standard curve was generated using a previously quantified *C. trachomatis* L2 cryptic plasmid positive control on each *C. trachomatis*-specific PCR run to allow quantification of transcripts. As described by Storm *et al* [23] the MIC was assigned to the lowest antimicrobial concentration where a ≥95% reduction in transcription was observed after a passage in the presence of antimicrobial (P1) in comparison with the initial inoculum (P0) for each strain. RT-PCR was used only for MIC assignment to negate subjectivity of immunofluorescent staining interpretation.

Statistical analysis

Geometric means of the azithromycin and doxycycline MICs were calculated and linear regression was used to analyse the relationship between the MICs and the different patient groups. As absolute MICs were not available for a number of isolates (i.e. MICs were ≤ or ≥) then MIC values a doubling dilution above or below the recorded MIC e.g. ≤0.064 mg/L was analysed as 0.032 mg/L and ≥1 mg/L was analysed as 2 mg/L). Fisher's exact test was used to compare azithromycin MICs ≤0.25 mg/L versus MICs >0.25 mg/L and doxycycline MICs ≤0.064 mg/L versus MICs >0.064 mg/L in the persistently infected and successfully treated patient groups respectively. Results for both tests were deemed significant if the p value was ≤0.05.

Results

Isolate retrieval

Isolates were retrieved from eleven patients with persistent *C. trachomatis* infections that met the inclusion criteria outlined previously (five in category 1, two in category 2 and four in category 3). In addition, isolates were retrieved from thirteen control patients with linked negative test-of-cure samples.

MIC data

Azithromycin MICs were ≤0.25 mg/L for 81.8% (9/11) of the isolates from patients with persistent infections and for 100% (13/13) of isolates from the successfully treated control patients (Table 2, Table 3). The azithromycin geometric mean MICs were 0.127 mg/L and 0.071 mg/L for isolates from the persistently positive group and the successfully treated patient group, respectively. Azithromycin MICs for two isolates (18.2%) in the persistently infected group were 2 mg/L and 0.5 mg/L (Table 2, Table 3). No difference (Fisher's exact test, $p = 0.3$; linear regression $p = 0.1$) was observed between the azithromycin MICs for isolates from the persistently infected patients compared with those for isolates from the successfully treated patients. The MICs of doxycycline for the isolates from the successfully treated patient group were significantly lower than MICs for isolates from the persistently infected patient group (Fisher's exact test, $p = 0.006$); doxycycline MICs for 7/11 (63.6%) isolates from patients with persistent infections were 0.125 mg/L, and for the remaining four isolates (36.4%) were ≥1 mg/L. The doxycycline MICs for most (7/13, 53.9%) isolates in the successfully treated group were ≤0.064 mg/L, at least two-fold lower than the lowest MICs for isolates from the treatment failure group. The doxycycline MICs for the five remaining isolates from the successfully treated group were 0.125 mg/L (3 isolates), 0.25 mg/L (1 isolate) and 1 mg/L (3 isolates) (Table 2). These patients were all treated with azithromycin 1 g only. The doxycycline geometric mean MICs were 0.322 mg/L and 0.097 mg/L for isolates from the persistently positive group and the successfully treated patient group, respectively ($p = 0.032$).

Assay reproducibility

To investigate the robustness of the susceptibility testing methodology, 11 (45.8%) isolates chosen at random (8 [8/11, 72.7%] from the persistently infected and 3 [23.1%] from the successfully treated patient groups) were repeat tested on the azithromycin assay and the MICs from both assay runs compared. All (11/11, 100%) repeat MICs were in complete agreement with initial testing. Thirteen (54.2%) isolates chosen at random (5 [5/11, 45.5%] from the persistently infected and 8 [8/13, 61.5%] from the successfully treated patient groups) were repeat tested on the doxycycline assay. Twelve (12/13, 92.3%) of the repeat MICs were in agreement with the initial MIC data. For one isolate in the successfully treated group the repeat MIC for doxycycline (≤0.064 mg/L) was at least four-fold (two dilution steps) lower than the initial MIC (≥0.25 mg/L). During initial validation of the assays the range of antimicrobial concentrations tested were altered as considered appropriate based on the MICs obtained. The initial assay for this isolate had an antibiotic range tested of 0.064 – 0.25 mg/L doxycycline whilst later assays were tested up to 1 mg/L doxycycline. Collectively 23/24 (95.8% [confidence interval: 76.9-99.8%]) of the isolates that were retested on either assay gave reproducible MIC.

Discussion

We have adapted and further developed an assay for phenotypic *in vitro* antimicrobial susceptibility testing of *C. trachomatis*. The assay was used to test clinical isolates sourced from two distinct patient groups, one with persistent *C. trachomatis* infections and the other with *C. trachomatis* infections successfully treated following first-line recommended therapy (i.e. 1 g stat azithromycin). Clinical isolates from both groups were assayed against azithromycin and doxycycline. The assay methodology produced reproducible MICs of both antimicrobials when isolates were retested, with 95.8% of isolates giving identical MICs. The exception was an MIC obtained in a 'failed' repeat assay that was at least four-fold lower than for the initial assay.

MICs of doxycycline for the isolates from patients who had persistent infections were significantly higher than for isolates from successfully treated patients. A number of the patients in the persistently infected group had been treated with doxycycline in addition to azithromycin (Table 2). The doxycycline MICs for these isolates varied from 0.125 mg/L, which is comparable to the majority of the MICs for isolates from the successfully treated group, to >1 mg/L which is significantly less susceptible. However, the doxycycline MICs for two isolates in the successfully treated group were also 1 mg/L and neither of these patients were treated with doxycycline regimens. The significance of these raised MICs is unclear.

There did not appear to be a difference between the MICs of azithromycin for isolates from the two patient groups. Indeed, whilst the majority of patients in the persistently infected group were treated at least twice with 1 g azithromycin stat regimens (Table 2) the MICs for the isolates from these patients were mostly within a two-fold dilution compared with the MICs for the isolates from the successfully treated patients and the 'susceptible' control strain, 314. There were two isolates in the persistently infected group with azithromycin MICs at least two dilutions higher than control strain 314 and the successfully treated patient group. Interestingly, both of these patients had only been treated once with 1 g azithromycin stat regimens. Overall, this suggests that the antibiotic pressure exerted by re-treatment with the same antibiotic did not select for increased MICs (reduced susceptibility) in these isolates. It may be hypothesised that heterotypic resistance induced *in vivo* may account for the similarity of MIC, but difference in clinical outcome seen with these patients if re-infection can truly be excluded, as asserted.

What is clear from the data presented is that much further work is needed to understand the relevance of the MICs obtained from both patients who resolve infection after treatment with first-line therapies and from patients who remain infected. *In vitro* susceptibility testing can only be performed with cultured isolates, which for *C. trachomatis,* are a rare commodity in the current diagnostic environment. Whilst molecular detection of known markers associated with antimicrobial resistance can infer genotypic susceptibility, emerging resistance can only be detected through *in vitro* susceptibility testing. Therefore access to isolates of clinically significant pathogens, such as *C. trachomatis,* is imperative.

For many organisms, such as *Neisseria gonorrhoeae*, there are internationally recognised standard protocols for antimicrobial susceptibility testing. No such standardisation exists for *C. trachomatis* [18] and antimicrobial susceptibility testing is particularly complex as it is an obligate intracellular organism requiring tissue cell culture for *in vitro* growth. [18] This, combined with the biphasic nature of the *C. trachomatis* lifecycle (where the extracellular phase is non-replicative), introduces a potential for assay variability not seen for other organisms. Suchland *et al.* (2003) and Wang *et al* (2005) described a range of factors that may influence the MICs for *C. trachomatis in vitro,* such as cell line used, inoculum size and time from where infection occurs to addition of the antimicrobial. Interpretation of the endpoint of the MIC assay can also be problematic. Traditionally, immunofluorescent staining of tissue cultures has been commonly used to identify aberrant chlamydial inclusions, but this method is time-consuming and subjective. In addition, failure to visualise *C. trachomatis* inclusions in *in vitro* cultures does not exclude a viable state that can proliferate once the antibiotic pressure has been removed. [27]

To negate subjectivity and to detect all viable organisms, we adapted a method previously described by Storm *et al.* (2005), which monitored the presence of mRNA transcripts in pre- and post-antimicrobial treated *C. trachomatis* cultures. Whilst the efficiency of reverse transcriptase PCR is known to be variable, the reproducibility of the MICs presented in this report indicate that this procedure was standardised as much as possible. The Storm assay was adapted to include detection of a predicted virulence factor gene on the *C. trachomatis* cryptic plasmid in place of the original *C. trachomatis omp2* gene. The cryptic plasmid is constitutively expressed throughout the *C. trachomatis* life-cycle and, whilst the number of copies of the plasmid carried can vary between different strains of *C. trachomatis,* [28] isolates were compared with themselves only. It was assumed that the plasmid copy number remained stable within a strain however it is possible that they may vary during different lifecycle stages and/or when challenged with antimicrobial; investigation of this was beyond the scope of this study but is a recognised potential limitation. We also increased the time that infected cultures were incubated prior to application of the antimicrobials from two hours, as described in Storm *et al*. (2005), to four hours to allow infections to establish more completely before challenge. Clean cell lines were screened for the presence of contaminants prior to inoculation as part of routine tissue culture maintenance and all infected cell line work was carried out in the presence of multiple antimicrobial/antifungal agents. However, as the isolates were clinical in origin it cannot be fully excluded that no other organism was present in the tissue culture at the time of susceptibility testing,

Despite development of a reproducible assay, there are limitations to this work. Interpretation of our MIC results was difficult as no susceptibility or resistance breakpoints exist for *C. trachomatis* and there is very limited data [29, 30] regarding the wild-type distributions of susceptibility to azithromycin and doxycycline for circulating strains. Due to the dearth of susceptibility data for this organism, how *in vitro* MICs correlate with treatment success or failure in the patient is poorly understood. Indeed, when the results presented in this report are taken into account i.e. evidence of consistent *in vivo* phenotypic resistance to azithromycin in the patients persistently infected with *C. trachomatis* without evidence of reduced susceptibility of the isolate *in vitro*, the picture becomes even more complex.

Given the move to the use of doxycycline as the preferred first-line therapy for NGU, in which *C. trachomatis* is the most commonly identified pathogen, [31] understanding the relevance of the raised doxycycline MICs in isolates from the persistently infected patient group is important. Particularly as doxycycline may in the future be given as prophylaxis to men who have sex with men as PrEP for bacterial sexually transmitted pathogens [32] and the impact of this increased doxycycline usage on *C. trachomatis* MICs is unknown. The high-level of assay reproducibility suggests that whilst the majority of strain MICs differed by only one doubling dilution, the difference (p=0.006) was unlikely related to the susceptibility testing methodology and an MIC shift towards less-susceptible was observed in the persistently infected patient group. However the root cause of this shift and its impact on clinical outcome is unclear. A larger observational case control study is required to generate data to allow appropriate antimicrobial stewardship. [33] This data may strengthen the case for recommendation of a test of cure in all patient groups.

In addition, a number of physiological factors, such as the host inflammatory response, that would form part of natural infection resolution (in addition to antimicrobial therapy) and individual patient pharmacokinetics that cannot be replicated in *in vitro* cell culture systems must also be considered. It is therefore difficult to hypothesise how representative an MIC alone would be as a marker of likelihood of treatment success. There are also few data available regarding how *in vitro* culturing of isolates affects the organisms' susceptibility to antimicrobials. The patients who were persistently infected with *C. trachomatis* were exposed to a minimum of two rounds of antimicrobial therapy, but viable organisms remained. These patients were thought unlikely to have been re-infected, but this cannot be excluded completely. Antimicrobial susceptibility assays were carried out secondary to the primary isolation from the clinical specimen. As a result, it was necessary to re-culture each isolate from an archived aliquot. It is possible that multiple passages in tissue culture in the absence of antimicrobial challenge could have affected the MIC obtained especially if surviving antimicrobial therapy in the patient led to a fitness cost. The authors recognise this as a weakness of the study and would recommend progressive processing through primary isolation and antimicrobial susceptibility testing to limit time in culture as an ideal. Further to this, the length, complexity and cost of the testing procedure is not amenable to large-scale phenotypic testing over a wide antibiotic concentration range. Processing of isolates from recovery from archive to obtaining MIC results took on average 15 working days. Nevertheless, we determined MICs of two therapeutically-relevant antibiotics for 24 clinical *C. trachomatis* strains. Any large-scale antimicrobial resistance surveillance in *C. trachomatis* would need to take advantage of molecular techniques to screen for genetic markers of reduced susceptibility in addition to *in vitro* susceptibility testing if reliable indicators could be identified. Reassuringly, in a recent large-scale genome sequencing study of global *C. trachomatis* isolates, no known molecular markers of antimicrobial resistance were detected. [34]

In summary, a reproducible method for phenotypic antimicrobial susceptibility testing of *C. trachomatis* has been described. The assay was employed for the analysis of a small number of clinical isolates from two groups of patients who had very different treatment outcomes. The azithromycin MICs for the majority of strains within the persistently-positive group were comparable with those for strains in the successfully treated group. However, the MICs of doxycycline were higher in the persistently infected than in the successfully treated patient group. Antimicrobial susceptibility testing and interpretation of elevated MICs in *C. trachomatis* is extremely challenging in the absence of breakpoints. Further work to generate wild-type and treatment failure distribution data should be undertaken.

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Transparency

The authors have no competing interests to declare.

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Table 1. Primer and probe sequences used for RT-PCR

	Sequence 5’-3’	Reference

Ct-Forward	GGA TTG ACT CCG ACA ACG TAT TC	Chen[26]
Ct-Reverse	ATC ATT GCC ATT AGA AAG GGC ATT	
Ct-Probe	FAM-TTA CGT GTA GGC GGT TTA GAA AGC GG-BHQ-1	
McCoy-Forward	TCA CCC ACA CTG TGC CCA TCT ACG A	Storm[23]
McCoy-Reverse	TGG TGA AGC TGT AGC CAC GCT	
McCoy-Probe	JOE-TAT GCT CTC CCT-(TAMRA)-CAC GCC ATC CTG CGT	
R19-Forward	CCT GCC GAA CTG AAA CAT CTT A	Modified from Pantchev[35]
R19-Reverse	CCC TAC AAC CCC TCG CTT CT	
R19-Probe	Cy5-CGA GCG AAA GGG GAA GAG CCT AAA CC-BHQ3	

Table 2. Summary of the characteristics of *C. trachomatis s*trains isolated from patients who were treatment failures (Pt.) or successfully treated (Ctrl).

				Treatment prescribed			MIC (mg/L)	
			omp1 genotype	Azithromycin 1 g stat	Doxycycline 100 mg bd 7 days	Other	Azithromycin	Doxycycline
Persistently infected	Cat. 1	Pt.1	G	X1	X2		≤0.125	0.125
		Pt.2	E	X2			≤0.125	>1
		Pt.3	E	X3	X1(14 days)		0.125	0.125
		Pt.4	E	X1	X1		2	0.125
		Pt.5	J	X2	X1	500 mg stat azithromycin then unknown dose od 4 days	≤0.125	0.125
	Cat. 2	Pt.6	D	X2			≤0.125	0.125
		Pt.7	G	X2	X1		≤0.125	0.125
	Cat. 3	Pt.8	E	X2			≤0.125	0.125
		Pt.9	E	X2			0.25	1
		Pt.10	E	X2	X1		≤0.125	>1
		Pt.11	E	X1		500 mg erythromycin qd 7 days	0.5	>1
	Successfully treated controls	Ctrl.1	F	x1			≤0.125	≤0.064
		Ctrl.2	E	x1			≤0.125	≤0.064
		Ctrl.3	E	x1			≤0.125	0.125
		Ctrl.4	E	x1			≤0.125	≤0.064
		Ctrl.5	E	x1			≤0.125	0.125
		Ctrl.6	D	x1			≤0.125	≤0.064
		Ctrl.7	E	x1			≤0.125	1
		Ctrl.8	F	x1			≤0.125	0.125
		Ctrl.9	E	x1			≤0.125	0.064
		Ctrl.10	D	x1			≤0.125	0.064
		Ctrl.11	D	x1			0.25	0.25
		Ctrl.12	E	x1			≤0.125	1
		Ctrl.13	E	x1			≤0.125	≤0.064

Control strains	314*	D				≤0.125	-
	R19[Δ]	N/A				-	≥1

* isolate from a successfully treated patient, [Δ] tetracycline resistant *C. suis* strain R19[25].

Persistently infected patients were categorised based on their likelihood of re-infection through a self-declared sexual behaviour questionnaire. Cat. 1 – no sexual contact since initial diagnosis, Cat. 2 – protected sexual contact only and Cat. 3 – unprotected sexual contact with a regular partner who had also tested positive and had been treated or a partner that did not test positive. Stat – statim, od – once daily, bd – bi-daily, qd – quarter-daily, N/A – not applicable.

Table 3. MICs of azithromycin and doxycycline obtained from isolates from two different *C. trachomatis*-infected patient cohorts.

		Persistently infected (n=11)	Successfully treated (n=13)	
Azithromycin	≤0.25	9	13	n°
		81.8	100	%
	>0.25	2	0	
		18.2	0	
Doxycycline	≤0.064	0	7	
		0	53.9	
	>0.064	11	6	
		100	46.2	

Geometric mean of MICs: Azithromycin - 0.127 mg/L (persistently infected) and 0.071 mg/L (successfully treated), Doxycycline – 0.322 mg/L (persistently infected) and 0.097 mg/L (successfully treated).